JOURNAL OF BACTERIOLOGY, Jan. 1996, p. 447–455 Vol. 178, No. 2
0021-9193/96/$04.00+0

Characterization of the *Erwinia chrysanthemi* Osmoprotectant Transporter Gene *ousA*

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Received 25 July 1995/Accepted 1 November 1995

Growth of *Erwinia chrysanthemi* in media of elevated osmolarity can be achieved by the uptake and accumulation of various osmoprotectants. This study deals with the cloning and sequencing of the *ousA* gene-encoded osmoprotectant uptake system A from *E. chrysanthemi* 3937. OusA belongs to the superfamily of solute ion cotransporters. This osmotically inducible system allows the uptake of glycine betaine, proline, ectoine, and pipecolic acid and presents strong similarities in nucleotide sequence and protein function with the proline/betaine porter of *Escherichia coli* encoded by *proP*. The control of *ousA* expression is clearly different from that of *proP*. It is induced by osmotic strength and repressed by osmoprotectants. Its expression in *E. coli* is controlled by H-NS and is *rpoS* dependent in the exponential phase but unaffected by the stationary phase.

Microbial pathogens encounter extremely diverse environments both inside and outside their hosts. In response to these adverse conditions, they undergo striking adaptations in order to survive and retain virulence. The growth of *Erwinia chrysanthemi*, which is involved in a systemic soft rot disease on a variety of higher plants, is influenced by desiccation (18, 21). We have recently analyzed the influence of osmotic strength on *E. chrysanthemi* growth and pathogenicity in the absence and presence of osmoprotectants (10). The consequences for pathogenicity were estimated by the effect of osmotic pressure on transcription of *pel* genes and pectate lyase activity. The transcription of the *pelE* gene, encoding the major extracellular pectate lyase enzyme, is induced in medium of high osmolarity, whereas the cellular growth rate was reduced. Osmoprotectants such as glycine betaine, proline, ectoine, and pipecolic acid were shown to be accumulated in the cells through an osmoinducible mechanism and stimulated growth. However, *pelE* transcription was reduced to basal levels.

Although uptake and accumulation of osmoprotectants have been observed in many bacteria, transporter structural genes have been characterized in only a few microorganisms. Considering the regulation of their transcription, a knowledge of which is essential to understanding osmoregulation, only the *proP* and *proU* operons of *Escherichia coli* and *Salmonella typhimurium* have been analyzed in depth (6–8, 13, 14, 20, 27, 28, 31, 32). The identification of structural domains involved in osmosensing and substrate binding could be improved by comparison of ProP and ProU with other osmoprotectant transporters in other bacteria. In this report, we characterize one of the osmoprotectant transporters of *E. chrysanthemi*, analogous to ProP of *E. coli*.

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MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The *E. coli* and *E. chrysanthemi* strains and plasmids used are described in Table 1. Cells were grown in LB or M63 glucose (0.2%) medium (33). *E. chrysanthemi* cells were usually incubated at 30°C, and *E. coli* cells were incubated at 37°C. The osmoprotectants choline, glycine betaine, proline, ectoine, and pipecolic acid were added at 1 mM to the growth medium. The osmotic pressure of M63 medium was increased by the addition of NaCl at appropriate concentrations and determined by measuring the freezing point with an osmometer. Bacterial growth was monitored by optical density measurements at 570 nm (OD_{570}). When required, antibiotics were added at the following concentrations: kanamycin (Km), 50 μg/ml; ampicillin (Ap), 50 μg/ml; and chloramphenicol (Cm), 20 μg/ml.

Transport assays. Cells grown to mid-exponential phase in M63 glucose medium were transferred to M63 glucose medium containing 0 or 0.3 M NaCl at an OD_{570} of 0.1 and reincubated. Cultures were stopped at an OD_{570} of 0.5 and harvested by centrifugation (15,000 × *g*, 5 min). Then they were resuspended to an OD_{570} of 1 in M63 medium containing 0 or 0.5 M NaCl. Transport assays and radioactivity counting were performed as described by Perroud and Le Rudulier (34). ^{14}C-osmoprotectants were produced as previously described (9, 19).

DNA manipulations. *E. chrysanthemi* was transformed by electroporation as described by Ausubel et al. (1), and chromosomal DNA was extracted by the method of Klotz and Zimm (22). The following procedures were carried out by the standard methods described by Sambrook et al. (37): preparation of plasmids and chromosomal DNA from *E. coli*, DNA ligation, bacterial transformation, agarose gel electrophoresis, and Southern blotting. The genomic DNA library of *E. chrysanthemi* used in this work was obtained from the Laboratory of Molecular Genetics of Microorganisms of Villeurbanne, CNRS URA 1486-1 (France). The library consists of *Sau*3A fragments ranging from 2 to 6 kb in size inserted into the dephosphorylated *Bam*HI site of pUC18. The mixture of recombinant plasmids was electrotransformed into competent *E. coli* MKH13 cells, and transformants were selected on M63 agar plates containing glucose (2 g/liter), ampicillin (50 μg/ml), NaCl (0.5 M), and osmoprotectants (1 mM).

Nucleotide sequence determination. The minimal 1.7-kb *Eco*RI-*Hin*dIII fragment obtained by exonuclease III deletion from the *Cla*I site of pECT1 that was able to complement *E. coli* MKH13 was inserted between the corresponding sites of pSU21, yielding pECT2-1 (Cm^r), and of pUC19, yielding pECT2-2 (Ap^r) (Fig. 1). These plasmids were subjected to exonuclease III digestion to generate smaller derivatives in order to sequence both DNA strands. DNA was sequenced with the Sequenase kit (US Biochemical Corp.). Reactions were carried out as recommended by the manufacturer. [α-^{35}S]dATP (1,000 Ci/mmol) was purchased from NEN Dupont. Homology searches were performed in the Swissprot (release 28) library.

Overexpression of OusA. A two-plasmid system based on the properties of the T7 RNA polymerase was chosen to study *ousA* expression (38). The minimal *E. chrysanthemi* DNA fragment able to complement MKH13, contained in pECT2-1 (Cm^r), was released by *Eco*RI and *Hin*dIII digestion and inserted between the *Eco*RI and *Hin*dIII sites of pT7-5 (Ap^r) and pT7-6 (Ap^r), yielding pT16230 and pT16015, respectively (Fig. 1). These two plasmids contained the insert in both orientations with respect to the strong T7 ϕ10 promoter. These

TABLE 1. Bacterial strains and vectors

Strain or plasmid	Relevant characteristics	Reference or source
E. chrysanthemi		
3937	Wild type	23
4037	Km^r *ousA*::*uidA*	This study
E. coli		
NM522	*supE thi* Δ(*lac-proAB*) *hsd5* (F′ *proAB lacI*^{q}*ZΔM15*)	30
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 flbB5301 rbsR*	4
RH90	MC4100 *rpoS359*::Tn*10*	17
PHL502	MC4100 *hns*::Tn*10*	P. Lejeune
GM50	MC4100 Φ(*proU-lacZ*) 3(λp*lac*Mu55)	28
GJ183	MC4100 Δ*putPA101 proP227*::Mu d1(Ap.*lac*)Δ(*pyr-76*::Tn*10*)	7
MKH13	MC4100 Δ(*putPA*)*101* Δ(*proP*)*2* Δ(*proU*)*608*	E. Bremer
K38	HfrC λ suppressor free	35
Plasmids		
pUC18	Ap^r	44
pUC19	Ap^r	44
pSU21	Cm^r	26
pUIDK1	Km^r Ap^r *uidA* cassette	2
pT7-5	Ap^r	38
pT7-6	Ap^r	38
pGP1-2	Km^r	38
pLKC480	Cm^r Km^r *lacZY* cassette	39
pLKC481	Cm^r Km^r *lacZY* cassette	39

hybrid plasmids were introduced into *E. coli* K38 (35), which contained the compatible plasmid pGP1-2, encoding T7 RNA polymerase under the control of the inducible λ p_L promoter and the gene encoding the thermolabile λ repressor cI857. The preferential labeling of the products of the genes cloned downstream of the ϕ10 promoter was obtained as previously described (40). Cell extracts were obtained by lysis in 60 mM Tris-HCl (pH 6.8)–1% sodium dodecyl sulfate (SDS)–1% 2β-mercaptoethanol and heating at 100°C for 10 min. Labeled products were analyzed by electrophoresis in a 12.5% polyacrylamide gel containing SDS (24) and revealed by autoradiography.

Construction of *ousA*::*uidA* fusion. The UIDK1 cassette (2) included a promoterless *uidA* gene that conserved its Shine-Dalgarno sequence. Insertion of this cassette in *ousA* in the correct orientation generated a transcriptional fusion and inactivated *ousA*. The *uidA*-Km cassette of pUIDK1 (Ap^r Km^r) was released by *Pst*I and *Bgl*II cleavage and ligated with *Nsi*I- and *Bcl*I-digested pECT2-1 (Cm^r) to yield pECT2-GUS (Fig. 1). This plasmid was transformed into *E. coli* MC4100, and Km^r Cm^r *uidA*$^+$ clones were selected. Analysis of the plasmids derived from transformants confirmed the *uidA*-Km cassette localization and orientation in pECT2-GUS. β-Glucuronidase activity was measured as described by Bardonnet and Blanco (2), and specific activity was expressed as nanomoles of *p*-nitrophenol liberated per minute per milligram of dry cell weight (DW).

Construction of *ousA::lacZ* fusions. *lacZ*-Km cassettes (39) were used to produce in-frame translational fusions, as deduced from the nucleotide sequence. The *lacZ* cassette of pLKC481 was released by *Nco*I digestion and inserted in the corresponding site of pECT1 to produce pL16377. The pLKC480 *lacZ* cassette was liberated by *Nru*I and ligated with *Nru*I-opened pECT1, yielding pL16385, or by *Eco*RV and ligated with *Eco*RV-digested pECT2-2, yielding pL304 (Fig. 1). These plasmids were introduced into *E. coli* MC4100, and Lac$^+$ Ap^r Km^r transformants were selected on LB plates containing 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml plus ampicillin and kanamycin. The blue colonies were picked up, and plasmid fusions were controlled. β-Galactosidase assays were performed as described by Miller (33), the cells being harvested in the mid-exponential phase of growth.

Disruption of the wild-type *ousA* gene of *E. chrysanthemi*. pECT2-GUS (Cm^r Km^r) bearing the *ousA::uidA* fusion was transformed by electroporation into *E. chrysanthemi* cells. Km^r Cm^s transformants were selected in order to isolate clones in which the *ousA::uidA* fusion had integrated into the host chromosome through homologous recombination. The recombination was confirmed by Southern blotting, and the resulting strain was named *E. chrysanthemi* 4037.

Nucleotide sequence accession number. The sequence reported here has been deposited in the EMBL database under accession number X82267.

RESULTS

Cloning of a 3.7-kb chromosomal region from *E. chrysanthemi* involved in osmoprotection. The structural gene for the osmoprotectant transporter was isolated by complementation of *E. coli* MKH13, which is deleted for *proP* and *proU* and for the proline porter gene *putPA*. Therefore, it is unable to grow in media of elevated osmolarity (0.5 M NaCl) in the presence or absence of osmoprotectants. MKH13 was transformed with an *E. chrysanthemi* genomic library carried by pUC18, and transformants were selected on minimal media containing 0.5 M NaCl and 1 mM glycine betaine, proline, ectoine, or pipecolate. Several clones were obtained on proline-containing medium only. All of them contained an identical plasmid, named pECT0, carrying a 3.7-kb insert (Fig. 1). ^{32}P-labeled pECT0, hybridized with the *Eco*RV-cleaved *E. chrysanthemi* chromosome, revealed two fragments, as expected from its restriction map (Fig. 1). One corresponds to the 2-kb internal *Eco*RV fragment of pECT0, and the other is one of the two *Eco*RV flanking fragments, the second one having too short a homology to be detected by the probe. In contrast, no hybridization with *Eco*RV-cleaved *E. coli* chromosomal DNA was observed.

The deletion of the 1.4-kb *Sal*I fragment of pECT0 yields pECT1, which was also able to complement MKH13 growth in the presence of osmoprotectants. Deleted derivatives were then produced from pECT1 by exonuclease III digestion from the *Cla*I site. The minimal insert able to complement MKH13 growth in minimal medium with 0.5 M NaCl and containing 1 mM proline was 1.7 kb in size. This fragment was subcloned into pSU21, yielding pECT2-1, in order to generate exonuclease III deletions from the *Sal*I site. A deletion as short as 0.1 kb suppressed proline protection of MKH13 growth at elevated osmolarity. Hence, the minimal region necessary for complementation was localized on the 1.7-kb DNA fragment of pECT2-1 and extended as far as the *Sal*I site. Cloned genes are expressed from their own promoter, since inversion of the insert orientation by cloning it in pUC19 (pECT2-2) did not affect its properties.

Nucleotide sequence analysis of the 1.7-kb DNA fragment. The nucleotide sequence of the 1.7-kb insert of pECT2-1 was determined as described in Materials and Methods. It contains a single open reading frame (ORF) of 1,497 bp, corresponding to a 54.74-kDa protein (Fig. 2). No typical signal sequence was observed at the beginning of this polypeptide. A putative ribosome-binding site (AAGG) was localized 11 bp upstream of the initiation codon, but no typical promoter was found before this sequence. The nucleotide sequence showed 74.3% identity with the sequence of *proP*, coding the *E. coli* proline/betaine porter, and the deduced amino acid sequence showed 83.2% identity and 93.6% similarity with the ProP sequence. In addition, among the proteins in the Swiss-Prot database, five transporters exhibit amino acid sequence identity higher than 15% with the deduced amino acid sequence. These proteins belong to the major facilitator superfamily and include alpha-ketoglutarate permease (KgtP) and the citrate-proton symports of *E. coli* (Cit1 and Cit2), *S. typhimurium* (CitA), and *Klebsiella pneumoniae* (Cit). The Blast comparison scores, based on statistical analysis, between these sequences were higher than 15%, suggesting that they are all homologous, i.e., derived from a common ancestor. We propose that the *E. chrysanthemi* cloned gene encodes an osmoprotectant transport system, named *ousA*, for osmoprotectant uptake system A.

Multiple alignments of these sequences revealed that their N-terminal parts are better conserved than their carboxyl parts (Fig. 2). In fact, the two osmoprotectant transporters OusA

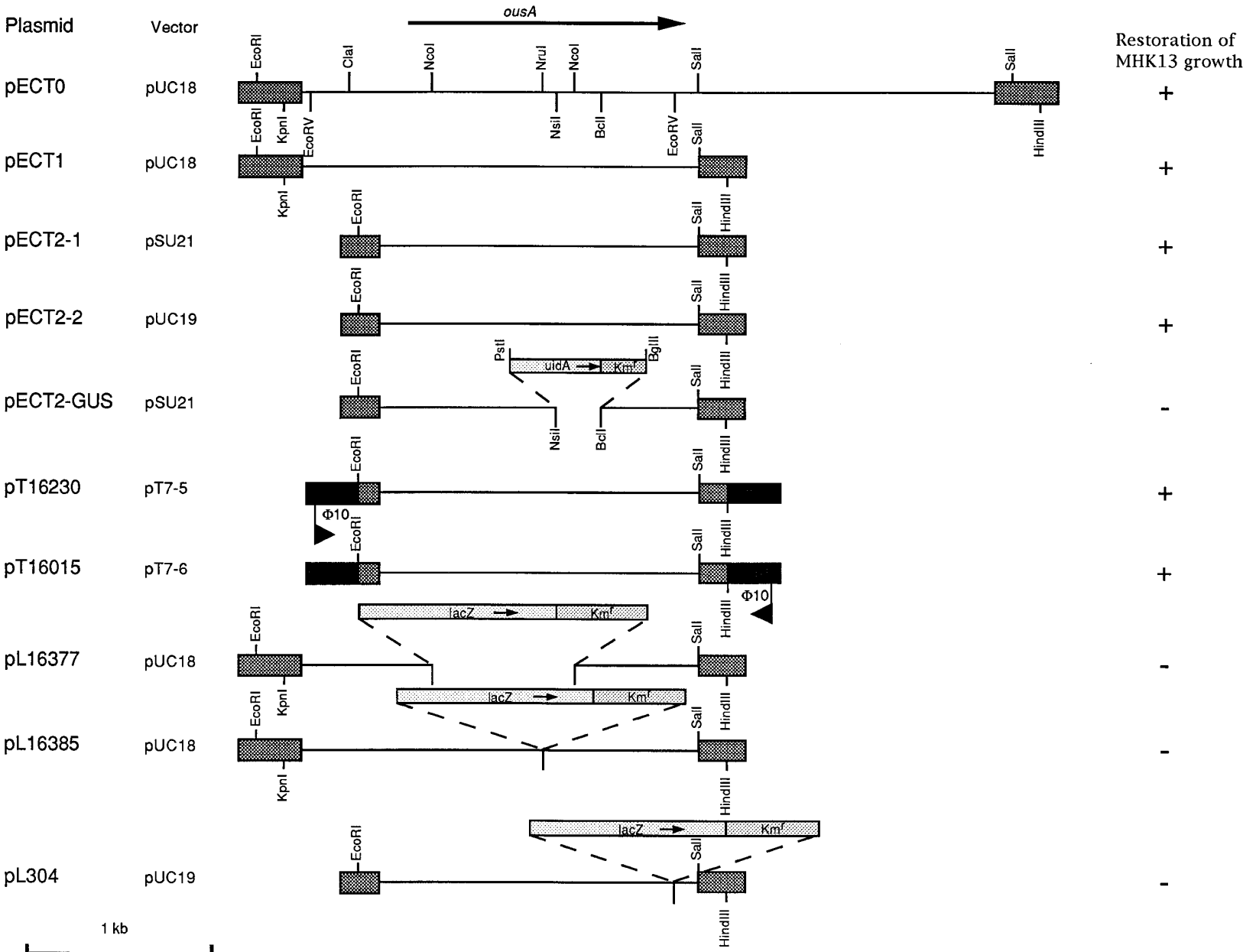

FIG. 1. Cloning and localization of *ousA*. *E. chrysanthemi* DNA is represented by the thin line; pUC18, pUC19, and pSU21 vector polylinkers are shown as shaded boxes; and pT7 vector polylinkers are shown as solid boxes. The exact position and direction of transcription of the *ousA* gene were inferred from the nucleotide sequence and are shown at the top of the figure. Only *E. chrysanthemi* DNA is drawn to scale.

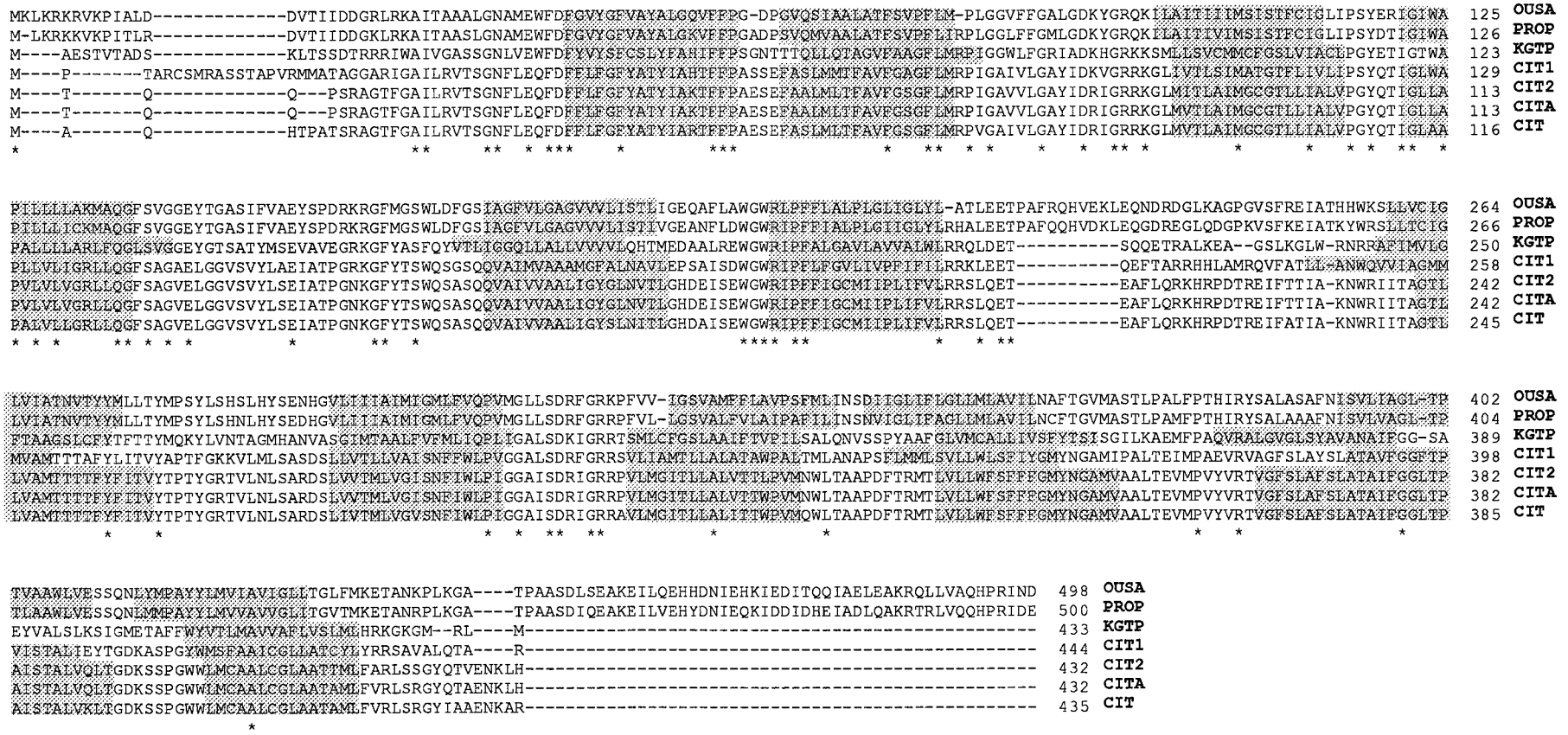

FIG. 2. Comparison of amino acid sequences and predicted topology of OusA and homologous proteins. Transmembrane domains are shaded, and identical amino acids in the seven sequences are marked with a star. OusA, osmoprotectant uptake system of *E. chrysanthemi*; ProP, proline/betaine transport system of *E. coli* (accession number P30848); KgtP, α-ketoglutarate permease of *E. coli* (P17448); Cit1 and Cit2, citrate-proton symports of *E. coli* (P07661 and P07680, respectively); CitA, citrate-proton symport of *S. typhimurium* (P24115); Cit, citrate-proton symport of *K. pneumoniae* (P16482). The multiple alignment was constructed by using the Treealign program (16). The standard parameters were used (mutation distance matrix derived from Doolittle's similarity measure; insertion/deletion weight $gk = 11 + 3k$).

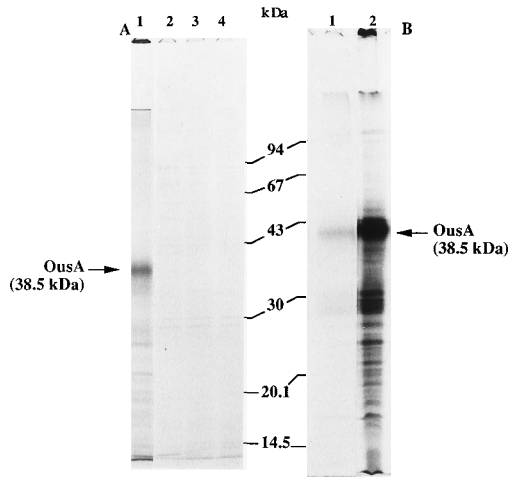

FIG. 3. Characterization of *ousA* product. (A) Autoradiogram of SDS-PAGE gel showing the [^{35}S]methionine-labeled proteins encoded by the pT7 constructions. Lane 1, pT16230, lane 2, pT7-5; lane 3, pT16015; lane 4, pT7-6. (B) Phase separation of labeled products with Triton X-114. The total protein extract produced by the overexpression of pT16230 was fractionated by phase separation with the nonionic detergent Triton X-114. The resulting fractions were subjected to SDS-PAGE and autoradiography. Lane 1, aqueous phase; lane 2, Triton X-114 phase. The positions of molecular mass standards are shown between panels A and B.

and ProP contain an extra C-terminal domain, composed of the last 50 amino acids, which is absent in the other homologous permeases. This domain was predicted to be able to form a coiled-coil structure and was proposed to sense and respond to reduced cellular turgor pressure (6). Topological prediction based on hydropathy analyses showed that these proteins have 12 transmembrane spans (Fig. 2), which is common to almost all members of the major facilitator superfamily (36). The fact that the loop regions were as strongly conserved as the transmembrane spans suggests that these sequences have not only a structural but also a functional importance.

Identification of *ousA* product. The minimal fragment containing *ousA* was released by *Eco*RI-*Hin*dIII double cleavage from pECT2-1 and cloned between the corresponding sites of the pT7-6 and pT7-5 vectors, yielding pT16015 and pT16230, respectively, in order to produce both orientations with respect to the T7 ϕ10 promoter. The plasmid-encoded gene products were synthesized in the presence of L-[^{35}S]methionine (Fig. 3). Only the orientation in pT16230, corresponding to the transcription direction of the predicted *ousA* ORF, allowed the expression of a single polypeptide with an apparent size of 38.5 kDa in SDS-PAGE. This value is similar to the apparent molecular mass of ProP (42 kDa) determined in an in vitro transcription-translation system (6), and, as for ProP, the apparent molecular mass is lower than that predicted from the nucleotide sequence.

Phase separation studies with the nonionic detergent Triton X-114 showed that most of OusA partitioned into the detergent layer, a behavior characteristic of integral membrane proteins (3) (Fig. 3). This location of OusA is not surprising, since the hydropathy profile is similar to that of ProP of *E. coli*. It presents various potential transmembrane domains, the amino- and carboxy-terminal domains being predicted to be cytoplasmic. OusA amino-terminal fragments of increasing length were fused to β-galactosidase. Three constructions were performed by introducing a *lacZ* truncated gene in frame with *ousA* at the 5′ *Nco*I site and at the *Nru*I site of pECT1, yielding pL16377 and pL16385, respectively, and the *Eco*RV site of pECT2-2, yielding pL304 (Fig. 1). As the three constructions had β-galactosidase activity, hybrid proteins were extracted with Triton X-114, and β-galactosidase was assayed in the aqueous and Triton X-114 phases. β-Galactosidase activity in the aqueous phase accounted for 100, 80, and 50% of global activity for fusions performed at *Nco*I, *Nru*I, and *Eco*RV, respectively. The remaining activity for the latter two fusions was present in the Triton phase. These results are not surprising, since the β-galactosidase moiety (116 kDa) is very big compared with OusA (54 kDa). This result suggests that the amino-terminal domain reaching to the *Nco*I location does not present any hydrophobic characteristics and, in the absence of a cleavable signal sequence, is certainly cytoplasmic, in agreement with the prediction.

Features of OusA. Glycine betaine, proline, pipecolic acid, and ectoine were chosen for their ability to restore growth to *E. coli* and *E. chrysanthemi* wild-type strains in high-osmolarity medium and were tested as osmoprotectants for MKH13 (pECT2-1) growth in M63 with 0.5 M NaCl. In the absence of an osmoprotectant, the growth of this strain was severely affected. Addition of 1 mM glycine betaine, proline, ectoine, and, to a lesser extent, pipecolic acid restored growth. MKH13 (pECT2-1) exhibited slow uptake for all the osmoprotective molecules when grown in M63 medium deprived of NaCl, but uptake was not affected by the osmolarity of the medium used for uptake measurements. V_{max} values of 1.1, 0.46, 3.43, and 0.038 nmol/min/mg DW were obtained for glycine betaine, proline, ectoine, and pipecolic acid, respectively, in assay medium with or without 0.5 M NaCl. Thus, OusA is not activated by osmotic pressure. On the contrary, uptake increased when the growth medium osmolarity was increased by adding 0.3 M NaCl (Table 2). V_{max} values increased and K_m values were nearly similar for all osmoprotectants. These results clearly showed that pECT2-1 encoded an osmoprotectant uptake system that was functional in *E. coli*.

The insertion of a *uidA*-Km^r transcriptional fusion cassette between the *Nsi*I and *Bcl*I sites of the insert in the direction of transcription, generating pECT2-GUS, suppressed osmoprotectant uptake in MKH13. In order to inactivate the corresponding gene in the *E. chrysanthemi* chromosome by homologous recombination, pECT2-GUS was introduced by electroporation into wild-type *E. chrysanthemi*. pECT2-GUS comes from pSU21, a p15A derivative vector which is not stably maintained in *E. chrysanthemi*. Thus, growth of Km^r Cm^s colonies results from the exchange of wild-type and mutated genes by homologous recombination in the recipient cell. Among 50 Km^r colonies analyzed, none presented a Cm^r phe-

TABLE 2. Characteristics of osmoprotectant uptake in *E. coli* and *E. chrysanthemi*[a]

Osmoprotectant	*E. coli* MKH13 (pECT2-1)		*E. chrysanthemi*			
			3937 (wild type)		4037 (*ousA*)	
	K_m (μM)	V_{max} (nmol/min/mg DW)	K_m (μM)	V_{max} (nmol/min/mg DW)	K_m (μM)	V_{max} (nmol/min/mg DW)
Glycine betaine	64	13	50	41	12	4
Proline	50	11	128	66	87	1
Ectoine	70	22	50	289	—[b]	—
Pipecolate	153	1	173	132	48	3

[a] Cells growing in M63 glucose medium with 0.3 M NaCl were harvested at an OD_{570} of 0.5 and resuspended in M63 with 0.5 M NaCl for uptake experiments. Values are means of triplicate determinations, and the error did not exceed 15%.
[b] —, not detected.

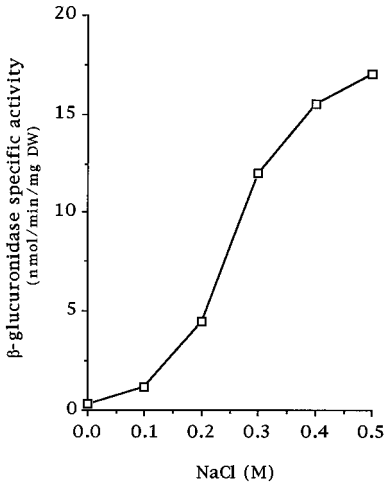

FIG. 4. Influence of medium osmolarity on *ousA* expression in *E. chrysanthemi*. Cells were cultivated in M63 medium with NaCl at increasing concentrations from 0 to 0.5 M and harvested at the end of the exponential phase of growth for β-glucuronidase assays. Results are the means of duplicate experiments; the standard deviation did not exceed 10%. The β-glucuronidase specific activity of the *ousA::uidA* fusion is expressed in nanomoles of *p*-nitrophenol liberated per minute per milligram DW.

notype. Thus, pSU21 was not present in the cell, either as a free DNA molecule or integrated into the chromosome. These results suggested that insertion resulted from a double crossover event, which was confirmed by Southern blotting analysis of *Bam*HI-cleaved total DNA of Km^r colonies with pSU21 and pECT2-GUS probes: pSU21 did not hybridize, and pECT2-GUS revealed restriction fragments characteristic only of its insert (data not shown).

The inactivation of the *E. chrysanthemi* chromosomal locus resulted in a faint phenotype variation. Glycine betaine, proline, pipecolate, and ectoine remained able to promote growth of the mutant in media of inhibitory osmolarity. Ectoine was less effective in the mutant than in the wild-type strain. Uptake parameters for these compounds were analyzed on wild-type and *ousA* mutant strains (Table 2). Only cells grown at high osmolarity were able to take up all the osmoprotectants, although a slight proline uptake was observed for cells grown in the absence of NaCl. These results suggest that uptake of osmoprotectants in wild-type and *ousA* mutant strains is inducible by high osmolarity. When wild-type cells were subjected to chloramphenicol treatment before salt addition, no uptake of glycine betaine, pipecolic acid, or ectoine was detected, whereas the omission of chloramphenicol treatment before transfer allowed the induction of uptake. Only proline possesses a constitutive transport system at low osmolarity (data not shown). The transport characteristics of wild-type (3937) and *ousA* mutant (4037) *E. chrysanthemi* strains were measured for cells grown on M63 glucose medium with 0.3 M NaCl. In the wild-type strain, the K_m values for the osmoprotectants were similar to those seen in *E. coli* MKH13(pECT2-1). The 4037 strain, in which *ousA* is inactivated, exhibited reduced K_m and V_{max} values for glycine betaine, proline, and pipecolate (Table 2). In contrast, ectoine transport was so poor that no uptake parameter could be estimated. These data suggest the existence of at least one other osmoinducible transporter involved in osmoprotectant uptake. The uptake parameters obtained for the wild-type strain result from the combination of at least two different transport systems with affinity values of the same order of magnitude for glycine betaine, proline, and pipecolic acid but different for ectoine.

Regulation of *ousA* expression. The transcriptional regulation of *ousA* expression was analyzed by monitoring β-glucuronidase activity in *E. chrysanthemi* 4037 carrying the chromosomal *ousA::uidA* fusion. Transcription of *ousA* increased with medium osmolarity (Fig. 4) but was not affected by growth phase (Fig. 5). β-Glucuronidase activity increased immediately after osmotic shock, reaching a maximal value in the early exponential growth phase and then remaining constant. Activity decreased with the onset of the stationary phase, a 50% loss of activity being observed after 24 h. When cells were grown in M63 medium containing 0.3 M NaCl and 1 mM glycine betaine, a transitory stimulation of *ousA* expression was observed (Fig. 5). The maximal level of β-glucuronidase activity, corresponding to 16% of that observed in the absence of an osmoprotectant, was reached in the early stages of exponential growth. The activity then decreased and was abolished at the end of the exponential growth phase. Identical results were obtained with proline, ectoine, and pipecolic acid (Table 3). The maximal β-glucuronidase activities observed were slightly greater than that obtained with glycine betaine.

When *E. coli* MC4100(pECT2-GUS) was grown in low-osmolarity medium, *ousA* expression was not affected by growth phase, since only a constant basal level of β-glucuronidase activity was observed during all growth stages (Fig. 6A). Immediately after osmotic upshift, β-glucuronidase activity increased, reaching a maximal level at the end of the exponential phase, and then remained constant. These results clearly shown that *ousA* expression is affected only by medium osmolarity and not by growth phase. Nevertheless, when pECT2-GUS was introduced into the MC4100 *rpoS* derivative strain RH90, we no longer observed the osmotic induction of *ousA* by

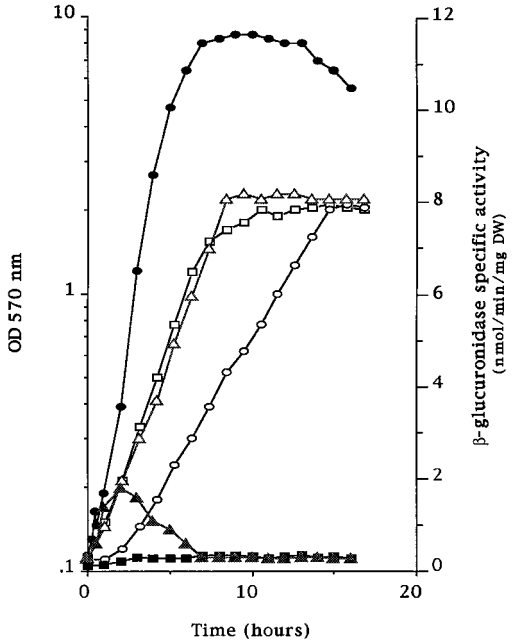

FIG. 5. Influence of osmolarity and glycine betaine on *ousA* expression during growth of *E. chrysanthemi* 4037 (*ousA::uidA*). The strain was grown in M63 medium with or without 0.3 M NaCl and 1 mM glycine betaine. β-Glucuronidase specific activity and OD_{570} were determined at various times during growth. Results are averages for at least three independent experiments. Open symbols, OD_{570}; solid symbols, β-glucuronidase activity. ■, □, 0 M NaCl; ●, ○, 0.3 M NaCl; ▲, △, 0.3 M NaCl plus 1 mM glycine betaine.

TABLE 3. Effects of medium osmolarity and osmoprotectants on *ousA* expression in *E. chrysanthemi* and *E. coli*[a]

NaCl concn (M)	Osmoprotectant	β-Glucuronidase activity (nmol/min/mg DW)	
		E. chrysanthemi 4037	*E. coli* MC4100 (pECT2-GUS)
0	None	0.3	2.2
0.3	None	11.5	15.0
	Glycine betaine	1.6	2.9
	Proline	3.2	4.7
	Ectoine	3.2	7.8
	Pipecolate	2.5	4.5

[a] Cells growing in M63 medium were harvested at the end of the exponential phase. β-Glucuronidase activity is expressed in nanomoles of *p*-nitrophenol liberated per minute per milligram DW. The results are the means of triplicate assays, and the error factor did not exceed 10%.

medium osmolarity (Fig. 6B). β-Glucuronidase specific activity remained at the basal level throughout growth whether the cells were grown at low or high osmolarity. Thus, like that of a few other genes (25), *ousA* expression is *rpoS* dependent in the exponential growth phase while being unaffected by stationary phase. This behavior is clearly divergent from that reported for *proP*, the expression of which is also *rpoS* dependent but increased at the end of exponential growth (42).

Osmoprotectants reduced the level of induction (Table 3) and provoked a constant decrease in *ousA::uidA* expression throughout growth. Ectoine was the less efficient, the β-glucuronidase activity obtained representing 50% of the maximal level observed in the absence of this osmoprotectant. The induction ratio and level of inhibition observed are reduced compared with those obtained in *E. chrysanthemi* (Table 3). This fact could be attributed to the high copy number of pECT2-GUS. Nevertheless, the regulation observed in *E. chrysanthemi* was also detected in *E. coli*, showing that the osmotic control machinery is conserved between the two strains and that the signals required for osmotic regulation of *ousA* are present on the 1.7-kb insert. Since translational *lac* fusions at *Nco*I, *Nru*I, and *Eco*RV sites showed the same regulation behavior as the *ousA::uidA* transcriptional fusion (data not shown), we concluded that *ousA* is regulated only at the transcriptional level and that all regulatory signals are located upstream of the *Nco*I site.

Cellular factors involved in *ousA* osmotic control. In attempt to determine if *proP* and *proU* of *E. coli* share any *trans*-acting regulation factors with *ousA*, pECT2-GUS was introduced into *E. coli* GJ183 (*proP-lac*) and GM50 (*proU-lac*). The presence of the high-copy-number plasmid did not modify the regulation of either *proP* or *proU*. β-Galactosidase activities were identical in transformed and wild-type strains and in cells grown in the presence or in absence of 0.3 M NaCl (data not shown). Thus, it seems either that *ousA* does not share any *trans*-acting factor with *proP* and *proU* or that the *trans*-acting factor(s) is not present at a concentration sufficient for the plasmid to limit its availability to *proP* and *proU*.

As H-NS could be considered an abundant factor involved in the regulation of various genes, particularly *proU* (8, 31, 41), pECT2-GUS was introduced into the MC4100 *hns* derivative PHL502. Under low-osmolarity conditions, the introduction of the *hns* mutation into MC4100 caused an increase in basal activity from 2.2 to 5.8 nmol/min/mg DW. After osmotic shock, we observed an immediate increase in β-glucuronidase activity in the early stages of exponential growth; maximal values of 15 nmol/min/mg DW for MC4100 and 17 nmol/min/mg DW for PHL502 were reached in the middle of exponential growth and remained constant. Thus, *hns* induced only an upshift in *ousA* expression without altering its osmotic induction pattern. Thus, as for the *proU* operon, H-NS is one of the factors controlling the osmoinducibility of *ousA* transcription.

DISCUSSION

The growth of *E. chrysanthemi* is affected by an increase in medium osmolarity, this effect being reversed by exogenously provided osmoprotectants. We showed that at least two uptake systems exist in *E. chrysanthemi*, both involved in the uptake of the four osmoprotectants glycine betaine, proline, ectoine, and pipecolic acid. This feature is identical to the system in *E. coli*, in which the osmoporters ProP and ProU are involved in the uptake of all known osmoprotectants (5, 11, 13, 19, 28, 29). ProU, the high-affinity binding-protein-dependent transport system, is encoded by the osmoinducible *proU* operon (12, 14, 27), whereas the *proP* gene, encoding the low-affinity transport

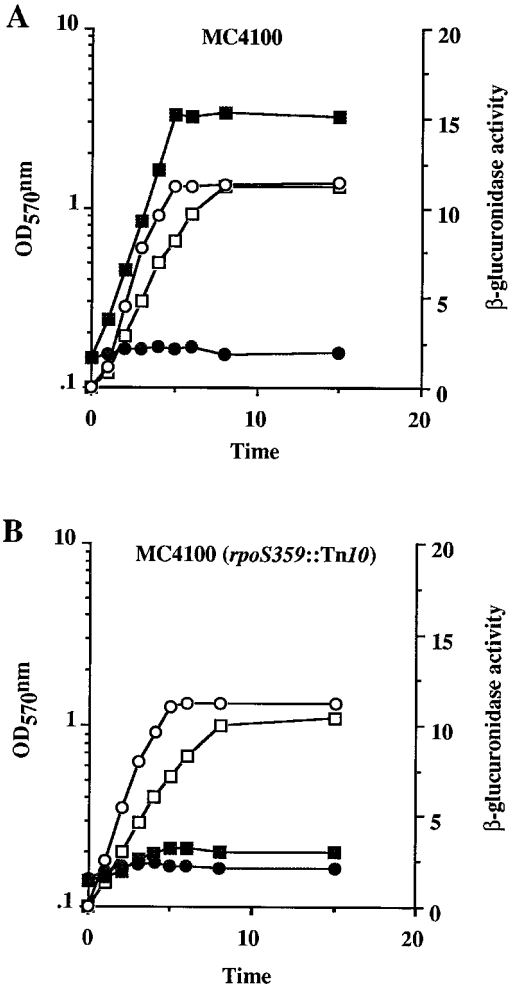

FIG. 6. Influence of medium osmolarity and *rpoS* mutation on *ousA* expression during *E. coli* growth. (A) MC4100(pECT2-GUS); (B) RH90(pECT2-GUS). The strains were grown in M63 medium with or without 0.3 M NaCl. β-Glucuronidase activity (expressed in nanomoles of *p*-nitrophenol liberated per minute per milligram DW) and OD_{570} were determined at various times during growth. Results are the averages for four independent experiments. Open symbols, OD_{570}; solid symbols, β-glucuronidase activity. ●, ○, 0 M NaCl; ■, □, 0.3 M NaCl.

system, has a constitutive expression which is enhanced by osmotic shock and amino acid limitation (15, 20, 28).

The *E. chrysanthemi* OusA transport system proved to be functional in *E. coli* and to be related to the ProP porter. These proteins have similar sequences, with a high conservation of residues and consequently also of predicted hydropathic profiles. These structural similarities are accompanied by common functional characteristics. ProP and OusA (i) show the same substrate specificity for glycine betaine, pipecolate, proline, and ectoine and (ii) are the major uptake systems involved in ectoine transport in their respective hosts. ProP also permits the transport of a large number of other osmoprotectants showing great structural diversity (11, 13). This ability should be analyzed for OusA in order to determine whether the few amino acid substitutions between OusA and ProP influence substrate specificity and/or affinity.

Two structural features differentiate ProP from the other members of the superfamily of solute ion cotransporters, i.e., an extended, central hydrophilic loop and an extended hydrophilic carboxyl-terminal domain that is likely to participate in the formation of an α-helical coiled coil or bundle (6). The structurally specialized carboxyl-terminal domain was suggested to be associated with the ability of the protein to sense and respond to reduced cellular turgor pressure (6). OusA also presents these two domains, but curiously, they constitute the two regions presenting the greatest divergence between OusA and ProP. In *E. chrysanthemi* cells grown at low osmolarity, no osmoprotectant uptake was detected whether transport activity was measured in media of low or high osmolarity. Since OusA protein is not activated by reduced cellular turgor, we suggest that amino acid variations in the carboxyl-terminal domain are responsible for the lack of OusA osmosensing.

The regulatory behavior of *ousA* and *proP* is different. In *E. coli* and *S. typhimurium*, *proP* induction by osmotic upshift is transient, and the steady-state level reached after adaptation is only twofold greater than that observed in the absence of osmotic stress, which is already relatively high (13, 20). The expression of *ousA* is clearly different from that of *proP* and is close to that of *proU* (20), being low in the absence of osmotic stress and stimulated by osmotic upshift, the maximal induction level being maintained throughout growth. This effect was observed in *E. chrysanthemi* as well as in *E. coli* bearing pECT2-GUS. The induction ratios, 30 and 7 in *E. chrysanthemi* and *E. coli*, respectively, were clearly greater than those of *proP* in *E. coli* and *S. typhimurium* (20). We did not observe an induction of *ousA* expression in the stationary phase as described for *proP* (32, 42); nevertheless, in *E. coli*, *ousA* expression is *rpoS* dependent in the exponential phase. This behavior is atypical of *rpoS*-dependent genes and has been described for a few genes, such as *xthA* (25). *ousA* is also negatively controlled by the *hns* product, while *hns* does not affect *proP* induction. The *hns* mutation enhanced the accumulation of σ^s in the logarithmic growth phase (43); thus, the increased basal level of *ousA* expression in *hns* mutant cells could result from a higher σ^s content or, as described for *proU* (41), from the absence of repression of *ousA* expression by the *hns* product. Nevertheless, other factors could be involved in *ousA* osmotic regulation, since *ousA* expression is osmoinducible in *hns* strains while the level of σ^s remains unchanged by osmotic induction (17).

All of these regulatory differences could be explained by the total lack of DNA homology upstream of the coding sequences of *proP* and *ousA*. Nevertheless, all of the osmoregulatory characteristics of *ousA* in *E. chrysanthemi*, including reversion of induction by osmoprotectants, were also observed in *E. coli*, suggesting common mechanisms of osmotic signaling in both organisms. We have shown that low-abundance *trans*-acting regulatory factors are not shared by *ousA* and *proP* or *proU*. Thus, *ousA* control is either performed by molecules in relative abundance in the cell or achieved by alterations of DNA topology or both. In *E. chrysanthemi*, factors like H-NS and σ^s could be involved in *ousA* expression. Mutants altered in *ousA::uidA* expression will be screened to identify regulatory signals and factors triggering *ousA* osmotic control.

ACKNOWLEDGMENTS

We thank E. Bremer, P. Lejeune, S. Reverchon, W. Nasser, and C. Gutierrez for providing strains and the *E. chrysanthemi* library and I. R. Booth for helpful comments and critical reading of the manuscript.

This work was supported by grants from the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de la Technologie, the Direction de la Recherche et des Etudes Doctorales, and the Région Bretagne (Programme BRITTA).

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